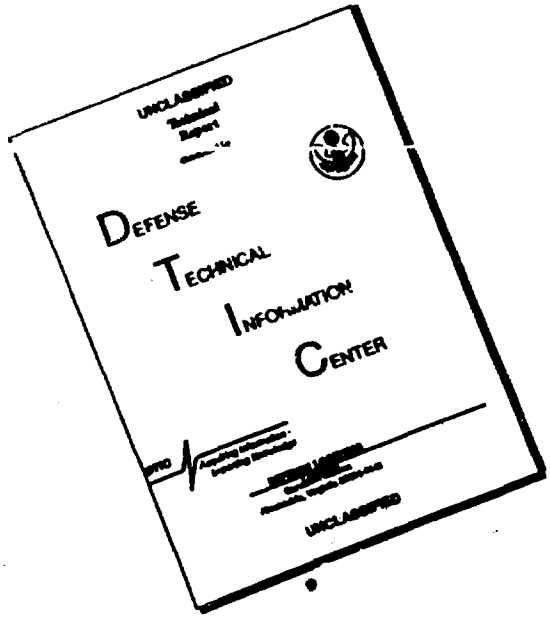
# 'STAPHYLOCOCCUS AUREUS' ENTEROTOXIN B RELEASE (EXCRETION) UNDER CONTROLLED CONDITIONS OF FERMENTATION

Joseph F. Metzger, et al

Army Medical Research Institute of Infectious Diseases
Frederick, Maryland

22 January 1973

# DISCLAIMER NOTICE




THIS DOCUMENT IS BEST QUALITY AVAILABLE. THE COPY FURNISHED TO DTIC CONTAINED A SIGNIFICANT NUMBER OF PAGES WHICH DO NOT REPRODUCE LEGIBLY.

APPLIED MICROBIOLOGY, May 1973, p. 770-773


Vol. 25, No. 5
Printed in U.S.A.

# *Staphylococcus aureus* Enterotoxin B Release (Excretion) Under Controlled Conditions of Fermentation

JOSEPH F. METZGER, ANNA D. JOHNSON, WILLIAM S. COLLINS II, AND VIRGINIA McGANN
*U.S. Army Medical Research Institute of Infectious Diseases, Frederick, Maryland 21701*

Received for publication 22 January 1973



Release of *Staphylococcus aureus* enterotoxin B (SEB) into the culture medium was initiated during the mid-log phase of growth. A medium consisting of 4% N-Z Amine A (Sheffield), 0.2% dextrose, and 1% yeast extract supported maximum production of SEB. Although pH of the medium during cultivation did not significantly affect the growth curve of the organism, the time required for detectable excretion was affected, as was the final yield. Optimal conditions for SEB production were achieved with pH control at 7.0; alkaline control (pH 8.0) produced only minimal amounts of toxin, whereas acid control (pH 6.0) resulted in 50% reduction in yield. Slightly less SEB was produced when there was no extrinsic pH control, and cultures were buffered only by media constituents and by-products of growth. With pH control at 7.0, deletion of 0.2% dextrose from the medium resulted in 40% reduction in the 8-h yield. There was also a delay in production during early stages of fermentation.



Release of staphylococcal enterotoxin B (SEB) into the culture supernatant fluid during cultivation of *Staphylococcus aureus* is affected by the culture medium and relative concentration of its constituents (6, 8, 10, 13, 15). Factors such as pH (1, 5, 12), aeration, and agitation (3, 10) also cause changes in the time of appearance and quantity of SEB released.

This paper describes experimental production of SEB in a fermentor system in contrast to previous studies which utilized shaker-grown cultures. Superior advantages include precise, continuous measurement and control of pH, temperature, agitation, and aeration. Such precise control of the latter three factors allowed independent evaluation of other parameters such as pH and medium constituents, which also influence the excretion of SEB.

## MATERIALS AND METHODS

**Microorganisms, media, and cultivation.** *S. aureus* strain 10-275, derived from strain S-6 for the production of maximal amounts of SEB, was employed. Stock inocula were prepared by lyophilizing 0.1-ml samples of 18-h growth harvested from Trypticase-soy-agar plates in 10% skim milk. A separate sample was utilized for each experiment. Lyophilized organisms were inoculated into 4% N-Z Amine A (Sheffield Chemical, Norwich, N.Y.) and 1% yeast extract-broth (Difco), and they were incubated for 18 h at 37 C in a shaking water bath. After a second passage in this medium, 100 ml of an 18-h culture was inoculated into a 50-liter volume of medium in the fermentor (Fermentation Design, Bethlehem, Pa.).

Media for the fermentor were designated as (i) AY medium consisting of 4% N-Z Amine A and 1% yeast extract-broth or (ii) AYD medium consisting of AY medium with 0.2% dextrose.

All cultures were grown at a constant temperature (37 C), automatic foam control, and agitation rate (400 rpm). Sterilized air was sparged through the culture at the rate of 10 liters/min.

Bacterial growth was estimated at hourly intervals by measuring the optical density of a 1:10 dilution of the culture at 540 nm. Plate counts of viable organisms were made concomitantly with turbidity measurements.

**Toxin assay.** Cultures were sampled at hourly intervals, and the concentration of SEB in supernatant fluid was estimated from measurements of radial immunodiffusion reactions in serum-agar (8). Antiserum was produced in goats by hyperimmunization with highly purified SEB and was incorporated in 1% agarose gel (Marine Colloids, Inc., Springfield, N.J.) adjusted to pH 8.3 with 0.063 M borate buffer in 0.033 M NaCl; serum-agarose gels 2.5 mm in thickness were prepared on glass microscope slides. A 1:30 dilution of antiserum permitted evaluation of 10 to 600 μg of SEB/ml, and a 1:100 dilution permitted evaluation of 1 to 20 μg of SEB/ml. Three-milliliter diameter reservoirs in the gel were filled with 10-μl samples of culture supernatant fluid or a reference standard of purified SEB; slides were incubated at room temperature

for 18 to 20 h, washed, dried, and stained with amido black. Standard curves were derived for each set of tests from a plot of $\log_{10}$ SEB concentration of reference solutions against the diameter (mm) of reaction. Reported values represent the arithmetic mean of 3 to 4 replicate determinations; individual values were within 10% of the mean.

## RESULTS

Immediately after inoculation, the optical density of cultures in the fermentor vessel was 0.010, the viable count was $3.0 \times 10^7 \pm 1.0 \times 10^7$ organisms/ml, and toxin concentration in the supernatant fluid was $1.46 \pm 0.51$ μg of SEB per ml. All fermentations were done in duplicate.

**Uncontrolled pH.** In preliminary trials, growth characteristics of strain 10-275 in AYD medium were examined in the absence of extrinsic pH control (Fig. 1). By 3.25 h after inoculation, maximum acidity developed (pH 5.8); by 4 h a decrease in acidity was noted, and by 10 h cultures were alkaline (pH 8.0). A fivefold increase in toxin concentration occurred in 4 h; a concentration of 440 μg of SEB/ml was achieved by 10 h. In these studies and in all subsequent experiments, toxin concentration at 24 h did not differ signigicantly from the 8- to 10-h values. During the period of initial, significant toxin release, viable counts increased from $8.7 \times 10^8$ to $1.5 \times 10^{10}$ organisms/ml. Optical density measurements paralleled viable counts.

**Constant pH.** (i) Extrinsic control of pH at 7.0 resulted in optimal yields of SEB. In AYD medium, SEB concentration tripled within 3 h and increased to 580 μg of SEB/ml by 10 h (Fig. 2); during this time viable counts increased from $1.0 \times 10^8$ to $1.1 \times 10^{10}$ organisms/ml. Optical density measurements again paralleled viable counts. In AY medium, SEB concentrations remained at, or below, 0-h levels for 3 h; by 4 h a 10-fold increase in SEB occurred with maximal concentrations of 255 μg of SEB/ml reached in 8 h (Fig. 3). Toxin release was unaffected by addition of 100 g of dextrose to AYD cultures after 5 h of fermentation.

(ii) At pH 6.0 initiation of toxin production in AYD medium was somewhat delayed (Fig. 4). A fourfold increase occurred in 4 h; the concentration at 10 h was 268 μg of SEB/ml. During this time, viable counts increased from $5.7 \times 10^8$ to $5.8 \times 10^9$ organisms/ml.

(iii) At pH 8.0 toxin concentrations remained unchanged for 7 h after inoculation, and less than a twofold increase was noted at 8 h (Fig. 5). Toxin yield at 10 h was 32 μg of SEB/ml. During toxin release, viable counts increased from $1.46 \times 10^8$ to $4.1 \times 10^9$ organisms/ml.

A summary of hourly production of SEB under various conditions is presented in Table 1.

## DISCUSSION

The time of production and quantity of SEB produced are affected by the type of medium and the concentration of its constituents. Maxi-

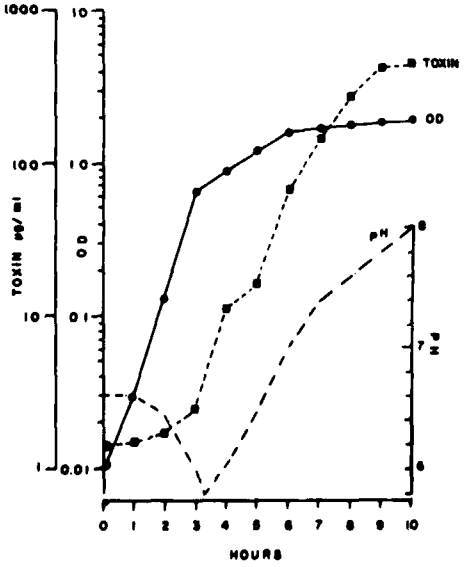



FIG. 1. *Fermentation with AYD medium without extrinsic pH control.*

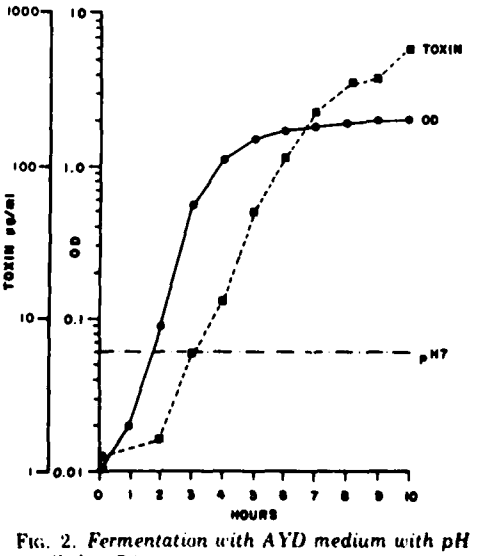



FIG. 2. *Fermentation with AYD medium with pH controlled at 7.0.*

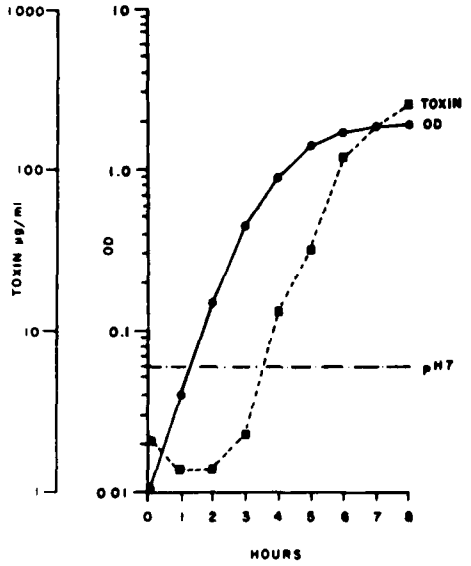



FIG. 3. *Fermentation with AY medium with pH controlled at 7.0.*

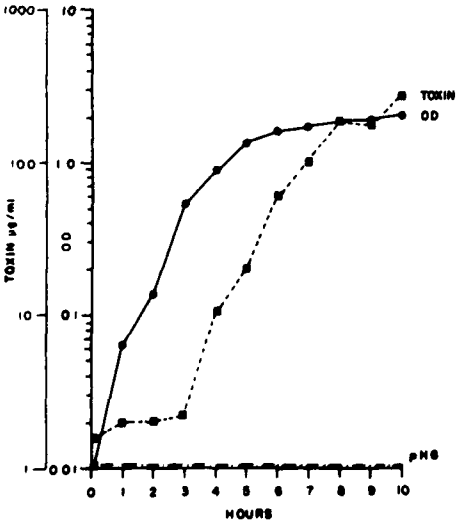



FIG. 4. *Fermentation with AYD medium with pH controlled at 6.0.*

mal production of SEB requires a high concentration of casein hydrolysates (2, 6). The medium, in addition to furnishing growth requirements necessary for production of SEB, provides adequate buffering capacity to prevent extreme acidity which may occur with the use of 1 or 2% protein hydrolysate media. By using a complete medium and rigidly controlled standard conditions, the effects of pH and dextrose addition could be studied independently of the effects of nutritional deprivation.

Morse et al. (12) found that addition of dextrose to a 1% protein hydrolysate medium caused a marked pH change during cultivation. Although the growth of the organism was not affected, there was a diminution in enterotoxin production. In our studies with a 4% protein hydrolysate medium, the inclusion of dextrose produced a lowering of the pH to 5.8 for a brief period, followed by a rapid return to neutral and later to alkaline. There was no disturbance in production of SEB, because pH rather than

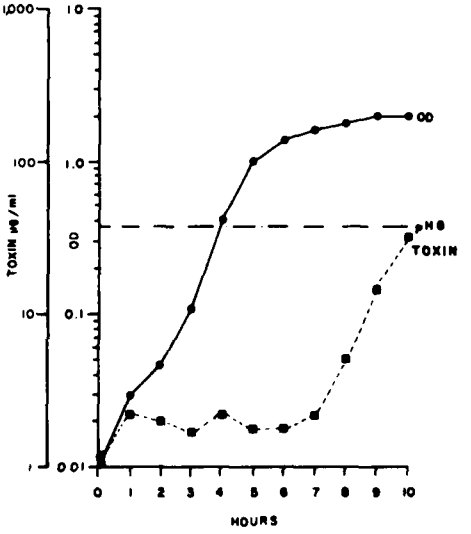



FIG. 5. *Fermentation with AYD medium with pH controlled at 8.0.*

TABLE 1. *Assays of toxin production during fermentation*

| Time (h) | Toxin (µg/ml) | | | |
|---|---|---|---|---|
| | Uncontrolled pH | pH 7 | pH 6 | pH 8 |
| 0 | 1.3 | 1.2 | 1.5 | 1.7 |
| 1 | 1.5 | 1.4 | 2.0 | 2.2 |
| 2 | 1.7 | 1.6 | 2.0 | 2.0 |
| 3 | 2.4 | 5.4 | 2.2 | 1.7 |
| 4 | 11.0 | 13.0 | 10.0 | 2.2 |
| 5 | 16.0 | 47.0 | 19.5 | 1.8 |
| 6 | 64.0 | 108.0 | 60.0 | 1.8 |
| 7 | 139.0 | 217.0 | 100.0 | 2.2 |
| 8 | 272.0 | 335.0 | 184.0 | 5.1 |
| 9 | 430.0 | 370.0 | 175.0 | 13.5 |
| 10 | 440.0 | 578.0 | 268.0 | 32.0 |

dextrose appeared to be the limiting factor in the release of enterotoxin. Optimal production of staphylococcal alpha toxin was found by Duncan and Cho (4) to require 0.2% dextrose in the medium. Addition of dextrose 5 h after inoculation of the fermentor with pH control at 7.0 did not inhibit release of SEB.

Optimal production of enterotoxin was achieved with AYD medium by controlling the pH at 7.0 (Table 1). Final viable cell counts revealed that at 10 h there was approximately 0.5-log less bacteria in either pH 6.0 or 8.0 controls. The pH 8.0 culture contained only minimal amounts of enterotoxin, and a significant amount was not produced until 8 h. At pH 6.0, fermentation produced only 45% of the toxin that was produced at pH 7.0.

In previous studies, the release of SEB into the culture supernatant fluid has been reported from mid-log to stationary phase (7, 8, 11, 12). Two factors can explain these discrepancies: differences in media constituents and the requirement for an accurate assay of enterotoxin in the microgram range.

The addition of 0.2% dextrose to medium rich in protein hydrolysate promotes good production of enterotoxin B. Significant amounts of SEB were produced when a medium consisting of 4% protein hydrolysate, yeast, and 0.2% dextrose was used. Cultures grown under these conditions produce enterotoxin in mid-log phase if pH is controlled at 7.0.

## LITERATURE CITED


1. Bergdoll, M. S. 1962. The chemistry and detection of staphylococcal enterotoxin. Amer. Meat Inst. Found., Circular 70, p. 47-53.
2. Bergdoll, M. S. 1970. Enterotoxins, p. 278-281. *In* T. C. Montie, S. Kadis, and S. J. Ajl (ed.), Microbial toxins, vol. 3. Academic Press Inc., New York.
3. Dietrich, G. G., R. J. Watson, and G. J. Silverman. 1972. Effect of shaking speed on the secretion of enterotoxin B by *Staphylococcus aureus*. Appl. Microbiol. **24**:561-566.
4. Duncan, J. L., and G. J. Cho. 1972. Production of staphylococcal alpha toxin. II. Glucose repression of toxin formation. Infect. Immunity **6**:689-694.
5. Genigeorgis, C., and W. W. Sadler. 1966. Effect of sodium chloride and pH on enterotoxin B production. J. Bacteriol. **92**:1383-1387.
6. Kato, E., M. Khan, L. Kujovich, and M. Bergdoll. 1966. Production of enterotoxin A. Appl. Microbiol. **14**:6.
7. McLean, R. A., H. D. Lilly, and J. A. Alford. 1968. Effects of meat-curing salts and temperature on production of staphylococcal enterotoxin B. J. Bacteriol. **95**:1207-1211.
8. Mah, R. A., D. Y. C. Fung, and S. A. Morse. 1967. Nutritional requirements of *Staphylococcus aureus* S-6. Appl. Microbiol. **15**:866-870.
9. Mancini, G., A. O. Carbonara, and J. F. Heremens. 1965. Immunochemical quantitation of antigens by single radial immunodiffusion. Immunochemistry **2**:235-254.
10. Markus, Z., and G. J. Silverman. 1968. Enterotoxin B production by nongrowing cells of *Staphylococcus aureus*. J. Bacteriol. **96**:1446-1447.
11. Markus, Z., and G. J. Silverman. 1969. Enterotoxin B synthesis by replicating and nonreplicating cells of *Staphylococcus aureus*. J. Bacteriol. **97**:506-512.
12. Morse, S. A., R. A. Mah, and W. J. Dobrogosz. 1969. Regulation of staphylococcal enterotoxin B. J. Bacteriol. **98**:4-9.
13. Schantz, E. J., W. G. Roessler, J. Wagman, L. Spero, D. A. Dunnery, and M. S. Bergdoll. 1965. Purification of staphylococcal enterotoxin B. Biochemistry **4**:1011-1016.
14. Schwartz, J. 1935. Variations du pH dans les milieux de culture destines a la production de toxine staphylococcique. C. R. Soc. Biol. **120**:1085-1086.
15. Wu, C. H., and M. S. Bergdoll. 1971. Stimulation of enterotoxin B production. I. Stimulation by fractions from a pancreatic digest of casein. Infect. Immunity **3**:777-783.